REMARKS

Claim 3 has been canceled herein, without prejudice or disclaimer. Claims 14 and 15 have been withdrawn from consideration as being drawn to non-elected species. Claims 1, 2, 4-11 and 13 are being considered in this application. Claims 1, 4, 6, 8, 10 and 13 have been amended. Reconsideration of the above identified application, in view of the above amendments and the following remarks, is respectfully requested.

Support for the amendments can be found in the specification as follows:

Claims 1 and 8: support is found on page 8, ll. 6-9 "...incubating such cells with a gp39 antagonist and **recipient** allogeneic or xenogeneic cells which are depleted of recipient T cells" and in original claim 1.

Claim 4: support is found in original claim 3.

Claim 10: support is found in Examples 1-9 of the application as filed.

Claims 6 and 13: support is found in original claim 1 and Examples 5-7 of the application as filed.

Objection to the Specification

The amendment filed September 22, 2003 was objected to under 35 U.S.C. §132 as allegedly introducing new matter, "5,876,718", into the specification. This objection has been rendered moot by cancellation of the alleged new matter as indicated in the current "Amendments to the Specification". Accordingly, Applicants kindly request withdrawal of this objection.

Rejection under 35 U.S.C. 103(a)

Claims 1-11 and 13 stand rejected as allegedly obvious over Noelle et al., U.S. Patent No. 5,876,718 ("Noelle") in combination with Riddell et al., 1990, J. Immunol Methods 128: 189-201 ("Riddell"), Rooney et al., U.S. Patent No. 5,962, 318 ("Rooney"), and Sykes et al., U.S. Patent No. 6, 006, 752 ("Sykes"). The Examiner alleges that Noelle combined with i) the irradiation of antigen presenting cells in Rooney, ii) the culture of donor T cells for treatment over varying lengths of time

in Riddell, and iii) the monitoring of the induction of T cell non-responsiveness *ex vivo* in Sykes render the claims of the present invention obvious.

Applicants respectfully traverse this rejection. In contrast to the Examiner's reasoning, the amended claims are unobvious over the combinations of references cited in the office action. The reasons for the failure of these references to render the claims obvious are that (1) Noelle, Rooney, Riddell and Sykes, alone or in combination, fail to teach or suggest all the limitations of the presently claimed invention, (2) there is no motivation to combine these references, and (3) even if these references were combined, the combined teachings might at best provide an "obvious to try" situation, but without reasonable expectation of success in this unpredictable art.

Noelle

Noelle discusses tolerance in connection with bone marrow transplantation in mice to inhibit Graft versus Host Disease ("GVHD") as occurring *in vivo*, not *ex vivo* (Example 5, col. 20 ,ll. 61-64):

> Treatment of mice at the onset of cGVHD with anti-gp39 (250 μg/mouse, days 0, 2, 4, and 6), reduce the number of leukocytes/spleen in cGVHD mice to values that were identical to mice without disease.

Moreover, Noelle discloses that *in vitro* treatment of the <u>recipient</u> with cGVHD with anti-gp39 is not effective. At col. 21, ll.11-14, it is stated that:

> The addition of anti-gp39 to cultures of spleen cells from mice with cGVHD did not reduce the levels of in vitro Ig production, suggesting that anti-gp39 was exerting its effects in vivo.

By contrast, the present claims are directed to tolerizing <u>donor</u> T cells *ex vivo,* followed by administration to the recipient of the donor T cells which have been tolerized *ex vivo*. There is no teaching or suggestion of this method in Noelle.

Amended claim 1, from which all pending claims depend, recites a method for inducing T cell tolerance or non-responsiveness of donor tissue containing T cells to desired alloantigen-

bearing cells from a recipient *ex vivo*. The Examiner's attention is kindly pointed to Examples 1 and 2 of the instant application. *See* pages 10 and 11 of the instant application.

In this Example, donor T cells, "highly purified CD4+ lymph node T cells", and alloantigen-bearing cells from a recipient, "C57BL/6 T cell depleted, irradiated spleen cells", were mixed. Anti-gp39 mAb was added to the mixture. Induction of T-cell tolerance or non-responsiveness was measured by analyzing the concentration of IL-2 in the supernatant of the cultured cells *ex vivo*.

Noelle does not disclose such a method. At best, Noelle describes an *in vivo* method for inhibiting acute and chronic GVHD in a recipient of allogeneic bone marrow transplantation with anti-gp39. This method is demonstrated in Example 5 of Noelle at columns 18-24. In particular, in this example, either chronic GVHD (cGVHD) or acute GVHD (aGVHD) is induced in mice (col.18, ll. 37-51). GVHD induced mice are treated with anti-gp39 antibody (e.g., col. 20, ll. 61-64) and inhibition of GVHD is monitored by detecting various responses in the mice (e.g., polyclonal Ig production *in vitro*, col. 19, ll.3-14). Noelle administers anti-gp39 to the recipient GVHD-induced mice in vivo, whereas the present claims disclose adding anti-gp39 antibody to a mixture of donor T-cells and alloantigen-bearing cells obtained from a recipient and treated *ex vivo*.

Furthermore, Noelle goes on to demonstrate that even though splenocytes from anti-gp39 treated mice with cGVHD produced levels of IgG_1 and IgA identical to mice without disease, "the addition of anti-gp39 to cultures of spleen cells from mice with cGVHD did not reduce the levels of in vitro Ig production, suggesting that anti-gp39 was exerting its effects in vivo." See col. 21, ll. 11-14).

The method of the present claims induces tolerance or non-responsiveness of donor T cells to alloantigen-bearing cells from a recipient completely *ex vivo* by mixing i) T cells from the *donor*; ii) irradiated alloantigen-expressing cells from the *recipient*; and iii) soluble CD40 or a soluble CD40 fusion protein. In contrast, Noelle's method involves the treatment of recipient cells with anti-gp39 antibody *in vivo*. Furthermore, Noelle demonstrates that *in vitro* administration of anti-gp39 antibody to spleen cells obtained from cGVHD induced mice does not "reduce the levels of in vitro Ig production." See, col. 21, ll. 11-14. Thus, not only does Noelle not teach or suggest the presently claimed invention, it goes as far as to teach away from the claimed invention.

Regarding the Examiner's contention that Noelle renders obvious the time ranges for culturing the T cells as per instant claims 6 and 7, and the method of assaying for IL-2 secretion from tolerized T cells as per instant claim 13, Applicants respectfully direct the Examiner's attention to the fact that absent a disclosure of tolerizing donor T cells ex vivo, Noelle cannot teach or suggest, or otherwise supply motivation to tolerize the T cells *ex vivo* for the time ranges presently claimed or to assay for IL-2 secretion from tolerized T cells.

Riddell

The claims at issue in the present invention teach methods for inducing T cell tolerance or non-responsiveness of donor T cells *ex vivo*. The Examiner combines Noelle with Riddell and contends that the combination teaches "...the growth and expansion of antigen-specific T cells in culture ... that can be employed for therapeutic use". However, Applicants respectfully note that Riddell does not teach tolerization of T cells *ex vivo*. Riddell merely stands for the proposition that a specific subset of antigen-specific T cells can be cloned and amplified in culture using anti-CD3 and anti-CD28 monoclonal antibodies. The cloned T cells can then be used in adoptive immunotherapy.

First, neither of the foregoing antibodies are used in the present invention, and second, the purpose of Riddell is to *induce* an immune response against a specific antigen (e.g., a tumor antigen), not to *prevent* an immune response to specific alloantigens (See, e.g., Riddell at abstract). By contrast, the antibodies against gp39 as described in the instant application are employed to block lymphocyte proliferation *in vivo,* which is completely the opposite of Riddell (whose goal is to induce and maintain proliferation *in vitro*). A reference which teaches an opposite concept teaches away, and cannot be properly combined to make an obviousness rejection. See *In re Lundsford*, 148 U.S.P.Q. 721, 726 (CCPA 1966).

Further, since Riddell is silent on the issue of inducing tolerance *ex vivo* and preventing graft-versus-host disease, one of ordinary skill in the art would not find motivation to combine Riddell and Noelle to arrive at the present invention (much less arrive at a specific set of time points for culturing T cells tolerized against alloantigens as in claims 6 and 7). Any combination of

Riddell with Noelle would appear to result in an exacerbation of transplant rejection by generation an army of transplant-specific T cells.

Rooney

The present invention discloses a method for inducing T cell tolerance or non-responsiveness of donor T cells to desired alloantigen-bearing cells *ex vivo*. In this method alloantigen-bearing cells can be treated to deplete recipient T-cells by irradiation. *See*, present claims 8 and 9. The Examiner combines Noelle with Rooney and contends that the combination teaches depleting antigen presenting cells of T-cells by irradiation. While Rooney does teach the irradiation of antigen presenting cells (APC), it teaches away from the claimed method of inducing T-cell tolerance of donor T-cells by a mixed lymphocyte reaction *ex vivo*. The focus of Rooney is similar to Riddell: to *stimulate* an immune response (CTL) to specific antigens for adoptive transfer, which is useful to treat infections in immunocompromised individuals, or to treat tumors. To achieve this, Rooney requires first "pulsing" an antigen presenting cell (APC) with a specific antigen, in the presence of effector cells (which include T cells), to generate a CTL response against the antigen. Rooney teaches that this method can be used to combat opportunistic infections, such as EBV, which commonly present in individuals who have received transplanted tissue. EBV infected lymphocytes can abnormally proliferate and result in a lymphoma. Transplanting into the recipient T cells that can specifically attack EBV-infected lymphocytes is therefore beneficial. Thus, Rooney teaches the opposite of tolerance induction.

Regarding the Examiner's contention that Rooney teaches irradiating the APCs to eliminate T cells, Applicants respectfully disagree with this as well. Rooney teaches irradiating the APCs following pulsing with antigen in order to prevent the proliferation of non-specific cells (i.e., dendritic cells that did not take up the antigen for presentation), not to deplete T cells. This ensures that *only* APCs specific for the desired antigen persist in culture (col. 15, ll. 2-5). Specifically, Rooney states that: "Irradiation of the antigen presenting cells prevents their proliferation, thus ensuring that only antigen-specific effector cells are selected in the culture". *See* column 15, lines 1-4. There exists no motivation to combine the irradiation of Rooney for depleting recipient T cells of Noelle.

Further, Rooney, like Riddell, fails to disclose the induction of tolerance of T-donor cells *ex vivo*. Accordingly, one of ordinary skill in the art would not find motivation to combine Rooney and Noelle to arrive at the present invention.

Sykes

The Examiner contends that the Sykes teaches determining the ability of T cells to release IL-2 in order to assess the effect of an immunosuppressive drug ("a help reducing agent") that is administered, e.g., prior to an organ transplant. While the Examiner is correct on this contention, this teaching does not bear on present claim 13, which is directed to a method of determining whether donor T-cell tolerance to a specific antigen has been induced by the claimed method. Sykes does not disclose the claimed assay of determining *ex vivo* donor T cell tolerance induction or non-responsiveness by measuring IL-2. Applicants respectfully note that Sykes teaches reduction of help of the recipient's T-cell (*emphasis added,* column 10, lines 47-51):

> "Help reduction", as used herein, means the reduction of T cell help by the inhibition of the release of at least one cytokine, e.g., any of IL-2, IL-4, IL-6, gamma interferon, or TNF, *from T cells of the recipient* at the time of the first exposure to an antigen to which tolerance is desired.

Furthermore, Sykes discourages using antibodies against T cells as the help reducing agent (*emphasis added,* column 10, lines 25-27):

> [A]nti-T cell antibodies, because they can eliminate T cells, are *not preferred for use as help reducing agents.*

Sykes teaches away the use of anti-T cell antibodies (*i.e.* the anti-gp39 antibody of the present claims) as drugs that inhibit the release of cytokines. Additionally, Sykes is silent on the issue of tolerance of donor T cells *ex vivo*. Therefore, one of ordinary skill in the art would not have been motivated to arrive at the claimed invention of assaying the inhibition of cytokines such as IL-2 (Sykes) by the anti-gp39 (i.e., anti-T cell antibody) (Noelle) for induction of donor T-cell tolerance *ex vivo*.

The Present Invention Describes an Unexpected Result in an Unpredictable Art

Applicants respectfully submit that it was both unpredictable in this art and unexpected that T cell tolerance or non-responsiveness of donor T cells can be effectively induced *ex vivo*, as noted on page 8, lines 6-9, of the specification itself.

First, the cited references teach away from the presently claimed invention, as described above. Second, even if the cited references were to be combined, they might at best render the invention "obvious to try," without reasonable expectation of success. However, "obvious to try" without reasonable expectation of success is not the standard under 35 U.S.C. § 103. The proper test requires determining what the prior art would have led the skilled person *to do*. The Examiner's attention is directed to the Federal Circuit's decision in *In re O'Farrell*, 853 F.2d 984, 7 USPQ2d 1673 (Fed. Cir. 1988). In particular, the court notes:

> [W]hat was "obvious to try" was to explore a new technology or general approach that seemed to be a promising filed of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.
>
> An "obvious to try" situation exists when a general disclosure may pique the scientist's curiosity, such that further investigation might be done as a result of the disclosure itself, but the disclosure does not contain a sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued.

The Examiner contends that Noelle teaches T cell non-responsiveness to desired alloantigens with anti-gp39 antibodies and antigen presenting cells *in vitro*, for transplantation before transfer of the transplant to a recipient *in vivo*. Applicants submit that Noelle, at best, gives only "general guidance" for inducing T-cell tolerance *in vitro* using anti-gp39 antibody. Noelle, either alone or when combined with the cited references, fails to contain sufficient teaching of how to obtain T-cell tolerance or non-responsiveness of donor T-cells to desired alloantigen-bearing cells *ex vivo*.

One of ordinary skill in the art would not have had a reasonable expectation that T-cell tolerance or non-responsiveness of donor T-cells to desired alloantigen-bearing cells would be

established by the administration of gp-39 antibody *ex vivo*. As indicated in the instant specification (at page 8, ll. 22-30), previous studies reported in the literature have demonstrated that there is no requirement that gp39 be present for the induction of *in vitro* T-cell activation. For example, Grewal *et al.* (Exhibit A) have shown that naïve T cells from mice genetically deficient in gp39 expression proliferated normally in response to polyclonal activators such as anti-CD3, conA, SEA, or SEB (shown in Exhibit A, Figure 3a, b, c and d, respectively). In addition, Grewal *et al.* tested for *in vitro* proliferative responses of lymph node $CD4^+$ T cells purified from Cyt-c-specific TCR transgenic mice lacking CD40L to Cyt-c, in the presence of APC from wild type mice and from CD40L-deficient mice. Proliferation of Cyt-c-specific $CD4^+$ TCR transgenic T cells from wild-type and from CD40L-deficient mice was identical (Exhibit A, Figure 3e). Grewal et al. teaches away in showing that T-cell activation *in vitro* does not require gp39, and indeed can occur normally in the absence of gp39. Therefore, the results demonstrated in the Examples of the present invention in which donor T cells are tolerized *ex vivo* by a mixed lymphocyte culture with anti-gp39 antibody, are truly unexpected and could not have been predicted from the cited art with a reasonable expectation of success.

Applicants respectfully submit that the invention of the amended claims is not rendered obvious by Noelle in combination with any or all of Riddell, Rooney and Sykes. Accordingly, Applicants respectfully request withdrawal of these rejections.

CONCLUSION

In view of the above amendments and remarks, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

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Respectfully submitted,

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Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand

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LACK of functional expression of CD40 ligand (CD40L) on T cells results in hyper-IgM syndrome (HIGM1), a human immunodeficiency associated with a severely impaired humoral immune response that is consistent with defects in B-cell responses[1–3]. Patients also succumb to recurrent opportunistic infections such as *Pneumocystis carinii* and *Cryptosporidial* diarrhoea[4,5], suggesting that T-cell functions are also compromised in these individuals, but so far this has not been explained. We have previously shown that mice deficient for CD40L, like HIGM1 patients, show grossly abnormal humoral responses[6]. Here we report that CD40L-deficient mice are defective in antigen-specific T-cell responses. Adoptively transferred antigen-specific CD4⁺ T cells lacking CD40L failed to expand upon antigen challenge of the recipients, showing that expression of CD40L on T cells is required for *in vivo* priming of CD4⁺ T cells and therefore for the initiation of specific T-cell immune responses.

To determine whether CD40L influences T-cell responsiveness, we immunized CD40L-deficient and wild-type mice with a protein antigen, keyhole limpet haemocyanin (KLH), and tested their *in vitro* recall proliferative response. A significantly reduced response was observed in CD40L-deficient mice compared with wild-type mice (Fig. 1*a*) and the antigen dose–response curve was shifted by several orders of magnitude. The same defect was demonstrated with two other protein antigens, hen egg lysozyme (HEL) and cytochrome *c* (Cyt-*c*) (Fig. 1*b*, *c*). Furthermore, a dramatic reduction in the production of both interleukin-4 (IL-4) and interferon-γ (IFN-γ) was seen in the CD40L-deficient mice (Fig. 1*d*, *e*), indicating a key role for CD40L in antigen-specific T-cell responses.

The reduced response in CD40L-deficient mice could either be caused by a problem with the antigen-presenting cells (APC) or with T cells. We therefore used APC from wild-type mice to activate CD4⁺-enriched T cells from CD40L-deficient mice immunized with KLH/CFA (Fig. 2*a*, *b*). Neither wild-type APC (Fig. 2*a*) nor lipopolysaccharide (LPS)-activated B cells (which express co-stimulatory activity and thus might bypass a CD40L requirement) were able to restore the defects (Fig. 2*c*). To address whether APC are intrinsically defective in CD40L-deficient mice, we adoptively transferred wild-type CD4⁺ T cells to CD40L-deficient mice; these transferred wild-type CD4⁺ T cells responded vigorously to KLH immunization in the CD40L-deficient recipient (Fig. 2*d*). These observations suggest that the *in vivo* defect lies at the level of the T cells rather than APC. Despite this defect, CD40L-deficient mice do make a weak proliferative response to antigens, suggesting that a CD40L-independent mechanism for T-cell activation must also exist *in vivo*.

To determine whether CD40L-deficient T cells have an intrinsic defect in the ability to proliferate, responses of naive T cells to polyclonal activators were analysed. Naive CD4⁺ T cells from both wild-type and CD40L-deficient mice proliferated normally in response to anti-CD3, concanavalin A (conA), SEA or SEB (Fig. 3*a–d*). To test the *in vitro* primary response to antigen, we crossed CD40L-deficient mice to a Cyt-*c*-specific-TCR transgenic mice, and obtained mice with T cells specific for Cyt-*c* but lacking CD40L. Proliferation of Cyt-*c*-specific CD4⁺ TCR transgenic T cells from wild-type and from CD40L-deficient mice was identical (Fig. 3*e*). Thus, the intrinsic potential of T cells to respond to antigens is preserved in CD40L-deficient mice. The number and ratios of various T-cell subsets in CD40L-deficient mice are normal, as shown earlier[6]. In addition, the ability to obtain functional Cyt-*c*-specific T cells from

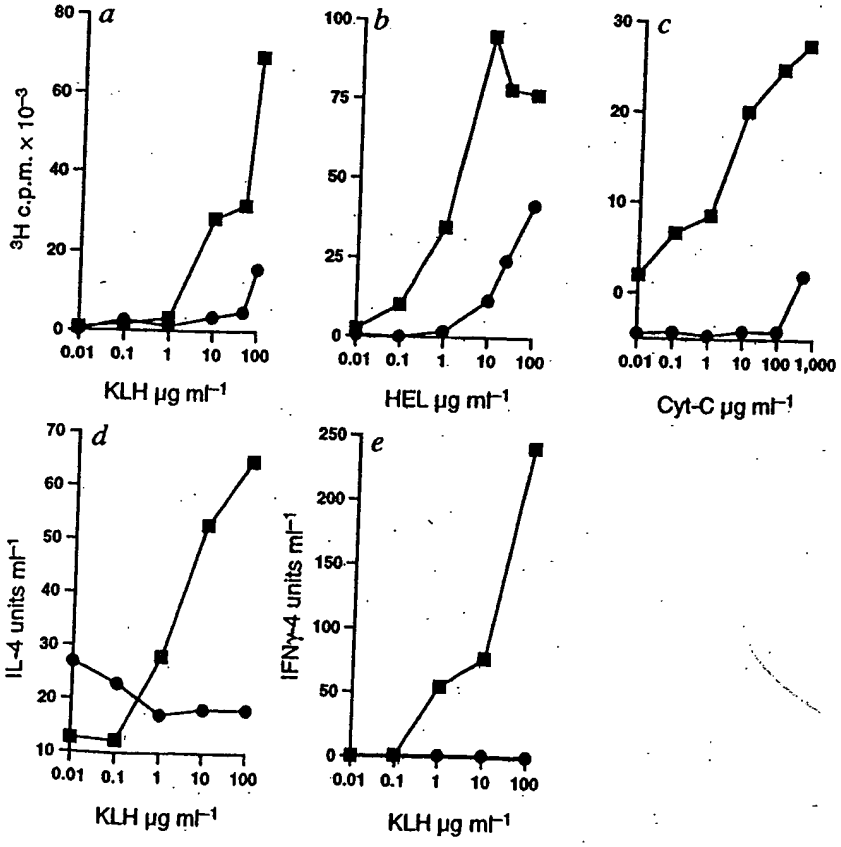

FIG. 1 CD40L mice have impaired lymph node recall proliferative responses to protein antigens. *In vitro* proliferative recall responses of wild-type (■) and CD40L-deficient (●) mice to KLH (*a*), to HEL (*b*), or to Cyt-c (*c*). Production of IL-4 (*d*) and IFN-γ (*e*) by purified CD4⁺ cells from draining lymph nodes of wild-type and CD40L-deficient mice immunized with KLH/CFA to *in vitro* challenge of KLH.

METHODS. Mice were immunized with 100 μg KLH, HEL or Cyt-c in complete Freunds adjuvant (CFA) in the hind footpads, and 9 days later draining lymph nodes were tested for recall proliferative responses as described[14]. CD4⁺ T cells were purified from draining lymph nodes as described[15]. Assays for cytokine production by T cells from KLH-immunized mice were conducted by culturing purified CD4⁺ T cells with APC in the presence of the indicated amount of KLH. After 4 days, supernatant IL-4 and IFN-γ was assayed using an ELISA kit as recommended by the manufacturer (Pharmingen). Experiments were repeated at least three times.

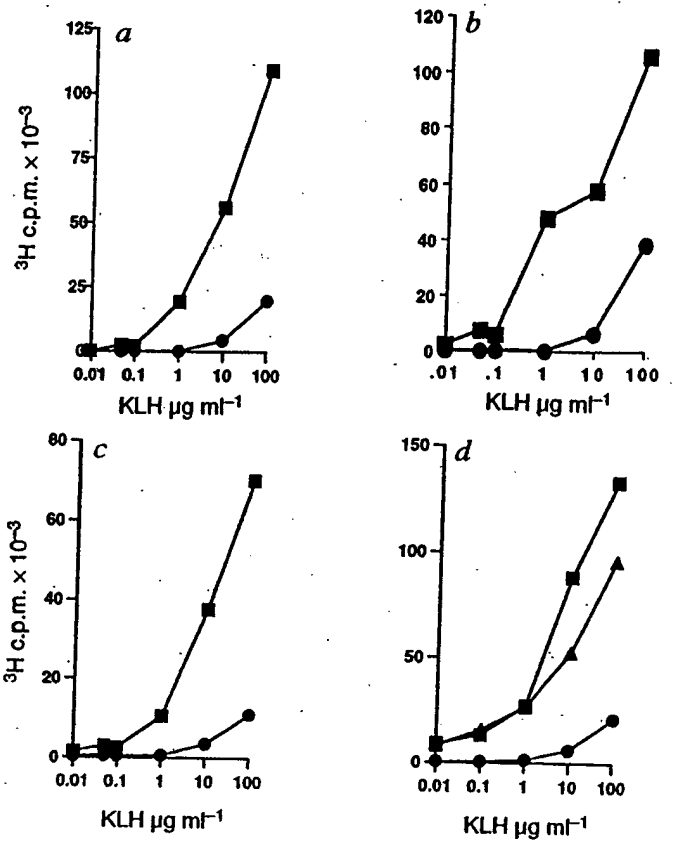

FIG. 2 Lack of response of CD40L-deficient mice to antigen immunization is due to a T-cell defect. Responses are shown of purified CD4$^+$ T cells from KLH/CFA-immunized wild-type mice (■) or CD40L-deficient mice (●) to KLH in the presence of control APC (*a*) or APC from CD40L-deficient mice (*b*); responses are also shown of purified CD4$^+$ T cells to KLH in the presence of LPS-activated splenic B cells as APC (*c*). *d*, Responses to KLH of CD40L-deficient mice (▲) that received 1×10^7 CD4$^+$ T cells i.v. from wild-type mice, and of wild-type and CD40L-deficient mice.

METHODS. Proliferation was measured by culturing 1×10^5 CD4$^+$ T cells from KLH-immunized mice with 5×10^5 irradiated (3,000 R) spleen cells from unimmunized wild-type or from CD40L-deficient mice or with LPS-activated (50 μg ml^{-1} for 48 h) B cells from wild-type mice in the presence of indicated amounts of KLH. Proliferation was determined after 4 d of culture by incorporation of ^{3}H-thymidine.

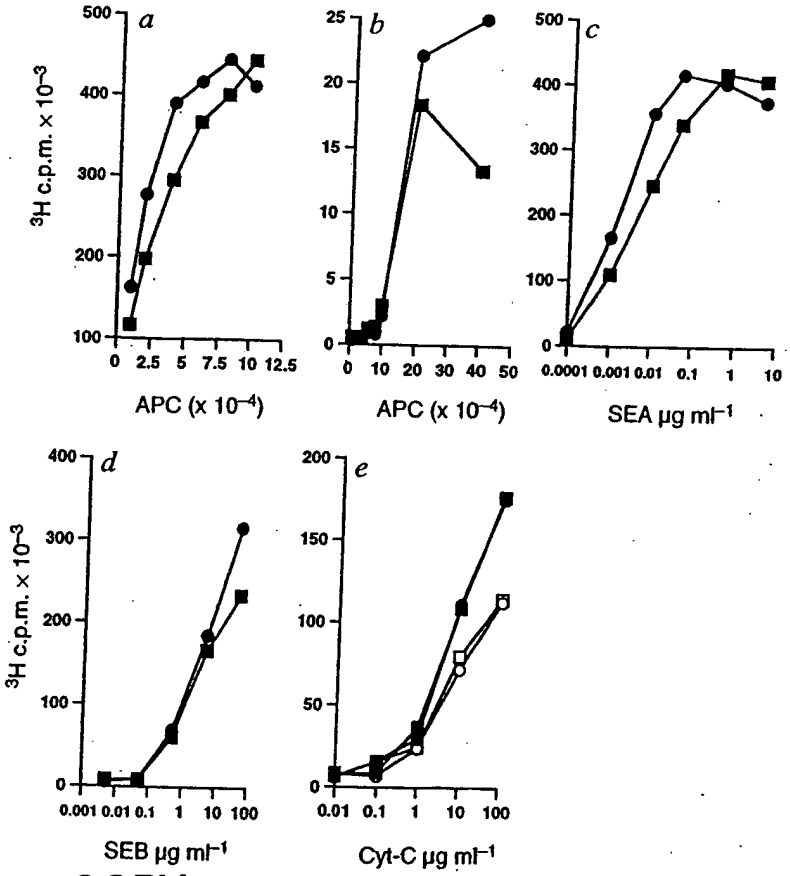

FIG. 3 Naive T cells from CD40L-deficient mice respond normally to antigenic and polyclonal stimuli. Lymph-node T cells purified from unimmunized wild-type (■) or CD40L-deficient (●) mice were cultured in the presence of *a*, conA; *b*, anti-CD3; *c*, SEA; or *d*, SEB. *e*, Lymph node CD4$^+$ T cells purified from Cyt-c-specific TCR transgenic mice lacking CD40L (circles) or control mice (squares) were tested for *in vitro* proliferative responses to Cyt-c in the presence of APC from wild-type mice (filled symbols), and from CD40L-deficient mice (open symbols).

METHODS. Naive lymph node cells were cultured in the presence of wild-type APC at different concentrations in the presence of conA (3 μg ml^{-1}) or anti-CD3 (1 μg ml^{-1}), and in the presence of various concentrations of SEA or SEB. Proliferation was determined after 4 days of culture by incorporation of ^{3}H-thymidine. For testing naive T cells from TCR transgenic mice, female mice homozygous for the CD40L mutation were crossed with Cyt-c-specific-TCR transgenic mice and progeny were screened for the presence of Cyt-c-specific TCR by staining with Vβ3 and Vα11 antibodies. Cyt-c-specific T cells from either CD40L-deficient or from wild-type control mice were cultured in the presence of APC from wild-type or CD40L-deficient mice together with different concentrations of Cyt-c. Proliferation was determined after 4 days of culture by incorporation of ^{3}H-thymidine.

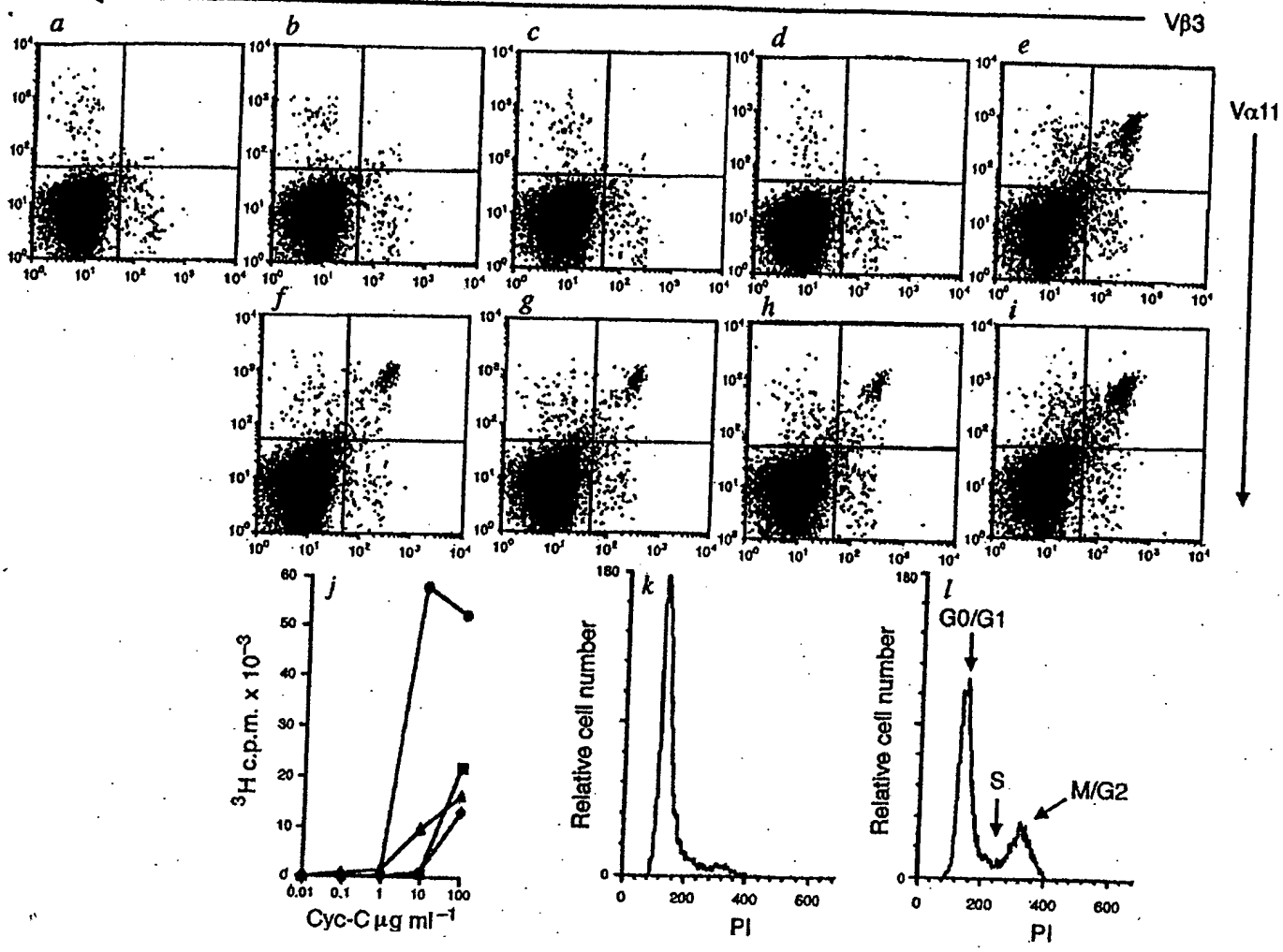

FIG. 4 Adoptive transfer of wild-type of CD40L-deficient Cyt-c-specific-TCR transgenic cells to recipient mice. FACS analysis of draining lymph node cells from mice that did not receive TCR transgenic T cells but were immunized with Cyt-c in CFA (*a*), and from mice that received 5×10^6 CD40L-deficient (*b*, *d*) or wild-type (*c*, *e*) TCR transgenic lymph node cells, and from mice that received 1.1×10^7 CD40L-deficient (*f*, *h*) or wild-type (*g*, *i*) TCR transgenic lymph node cells are shown. Recipient mice were also immunized with Cyt-c in CFA (*d*, *e*, *h*, *i*). *In vitro* recall responses of draining lymph nodes to Cyt-c (*j*) from mice that received 1.1×10^7 CD40L-deficient (◆) or wild-type (■) TCR transgenic lymph node cells, and from mice that were immunized with Cyt-c in CFA and also received 1.1×10^7 CD40L-deficient (▲) or wild-type (●) TCR transgenic lymph node cells. *k*, Flow cytometry plots of cell-cycle analysis for draining lymph node cells by propidium iodide staining for mice that received 1.1×10^7 TCR transgenic lymph node cells lacking CD40L and were immunized with Cyt-c/CFA, and *l*, mice that received 1×10^7 wild-type TCR$^+$ T cells and were immunized with Cyt-c/CFA.
METHODS. Cyt-c TCR transgenic lymph node cells from mice lacking CD40L or from control mice were adoptively transferred i.v. into recipient mice (TCR-negative littermates). A set of recipient mice were also immunized with Cyt-c in CFA (100 μg per mouse in the hind footpads). Five days after transfer, draining lymph node cells were stained with Vβ3 and Vα11 antibodies. Recipient mice that received TCR transgenic lymph nodes but were not immunized with Cyt-c, and mice that did not receive TCR transgenic cells but were immunized with Cyt-c were also tested as controls in the experiments. *In vitro* proliferation of draining lymph node cells was also measured in the presence of Cyt-c. Cell cycle monitoring was as ref. 16.

TCR transgenic CD40L-deficient mice indicates that positive selection of transgenic TCR-specific T cells does not require CD40L.

The failure of the apparently functional T cells from CD40L-deficient mice to respond to antigen stimulation *in vivo* might be explained by an inefficient *in vivo* priming of antigen-specific T cells in CD40L-deficient mice. We therefore adoptively transferred a limiting number of T cells from Cyt-*c*-specific-TCR transgenic mice into recipients immunized with Cyt-*c* (ref. 7) and examined their draining lymph nodes for the presence of transgenic TCR-specific T cells (Fig. 4*a–i*) and for Cyt-*c*-specific T-cell responses (Fig. 4*j*) five days after transfer. T cells lacking CD40L failed to expand *in vivo* upon antigen challenge, whereas wild-type cells expanded normally (Fig. 4*a–i*). The same results were seen when expansion was monitored 3 days postimmunization (data not shown). To test whether the numbers of T cells from CD40L-deficient mice had decreased after adoptive transfer (for example as a result of apoptosis or a graft-rejection artefact), we repeated the experiments using a higher dose of input T cells. Again, expansion was seen for wild-type CD4$^+$ T cells but not for the CD40L-deficient T cells. The initial population of transferred cells does not, however, diminish, suggesting that CD40L-deficient T cells fail to expand but do not die. Consistent with this, the *in vitro* T-cell response of CD40L-deficient T cells recovered from draining lymph nodes, which is not defective (Fig. 3*e*), can easily be seen for the adoptively transferred CD40L-deficient cells and at an expanded level for wild-type CD4$^+$ T cells (Fig. 4*j*). To determine the stage at which the numbers of CD40L-deficient T cells fail to expand, adoptively transferred Cyt-*c*-specific TCR T cells were analysed for entry into the cell cycle. Wild-type TCR$^+$ T cells enter the cell cycle and substantial numbers of cells (35.5%) were seen in the S/M/

G2 phases of the cell cycles, with the remaining cells in G0/G1 phase (64.5%) (Fig. 4*l*), whereas most (89%) TCR^+ cells from CD40L-deficient mice were in G0/G1 phases, with few cells entering S/M/G2 phases (11%) (Fig. 4*k*). This suggests that the defect that leads to failure of the clonal expansion of T cells in CD40L-deficient mice is at an early stage of T-cell activation, preventing T cells from entering the cell cycle.

How then does the CD40–CD40L interaction regulate T-cell priming? This interaction is required for the induction of co-stimulatory activity in B cells[8–10]. We have recently confirmed that wild-type, but not CD40L-deficient T cells can activate co-stimulatory activity in B cells *in vitro*[11]. We believe, however, that this mechanism is unlikely to explain the failure of T-cell priming in CD40L-deficient mice. First, it has recently been shown using B-cell-deficient mice that B cells are not required for T-cell priming to KLH, one of the antigens used in our study[12]. Thus, CD40L activation of B-cell function would not be required for the T-cell clonal expansion we have studied, and therefore could not explain the deficiency in CD40L-deficient animals. Second, the same deficiency in T-cell priming is evident in immunization of myelin basic protein (MBP)-specific TCR transgenic mice with a short peptide of MBP which provokes experimental allergic encephalomyelitis (EAE) in control but not CD40L-deficient animals (I.S.G. and R.A.F., unpublished observations); T-cell response to peptide antigens requires dendritic cell but not B-cell APC function[13]. One possibility still to be investigated is that the CD40–CD40L interaction is necessary for the activation of co-stimulatory activity in APC such as dendritic cells, which are believed to initiate the immune response.

In summary, our results suggest that CD40L is required for *in vivo* clonal expansion of antigen-specific T cells, which in turn may explain the susceptibility of HIGM1 patients to *Cryptosporidial* and *Pneumocystis* infections. Likewise, CD40L-deficient mice have an impaired ability to resist *Leishmania* infection, which is probably contained by T-cell response leading to macrophage activation (L. Soong *et al.*, manuscript submitted). Agonists or antagonists of the CD40–CD40L interaction may eventually have therapeutic benefits: for example, an agent directed at the function of CD40L might be useful in the treatment of autoimmune diseases such as multiple sclerosis. □

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CD40 ligand-transduced co-stimulation of T cells in the development of helper function

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Mice that lack either CD40[1,2] (expressed on B cells) or CD40 ligand[3,4] (expressed on activated T cells) are able neither to make IgG, IgA or IgE antibody responses, nor to generate germinal centres (the sites of formation of memory B cells). It has been assumed that these lesions were the result of an absence of signals to B cells through CD40. Here we show that the failure to signal T cells through CD40 ligand is an important contributory cause. Administration of soluble CD40 *in vivo* to CD40 knockout mice, restoring the missing signal through CD40 ligand, initiates germinal centre formation. Furthermore, T cells primed in the absence of CD40 (in CD40 knockout mice) are unable to help normal B cells to class switch or to form germinal centres (GC). These results indicate that co-stimulation of T cells through CD40 ligand causes their differentiation into cells that help B cells to make mature antibody responses and to generate memory populations.

To test the ability of T cells primed in the absence of CD40 to help B cells, we immunized CD40 knockout mice, and normal littermates, with keyhole-limpet haemocyanin (KLH). Seven days later, purified T cells from these mice were injected into lightly irradiated CD40 knockout mice, with B cells from non-immune, CD40-expressing, IgH^a congenic donors. These allotype-distinct 'indicator' B cells enable the donor/recipient source of serum antibodies to be determined. Adoptive hosts were immunized with dinitrophenylated KLH (DNP-KLH) (Fig. 1). Ten days after cell transfer, the mice that had received T cells primed in $CD40^+$ environment produced anti-DNP IgM, IgG1, IgG2a and IgG2b antibodies, and formed GC in their spleens. Mice that received T cells primed in CD40 knockout mice produced only IgM antibodies and exhibited no splenic GC (Fig. 1). The IgM and IgG antibodies were of donor IgH^a allotype. The response to DNP-KLH is T-dependent as athymic, nude mice make no anti-DNP or anti-KLH antibodies (results not shown). Thus, T-dependent IgM production can proceed in the absence of CD40 signals to B cells[1,2] and without full-blown helper activity. As full helper activity does not develop over the 14 days in the presence of $CD40^+$ B cells in the adoptive hosts it seems that the CD40–CD40-ligand interaction is crucial during the T-cell priming event and, possibly, cells other than B cells are required.

It is possible that T cells from $CD40^{-/-}$ mice are not tolerant of the $CD40^+$ donor B cells and might therefore reject them. We find no evidence for this as donor IgM^a serum responses were made and IgH^a-positive B cells could be detected in the spleens of recipient mice (results not shown). Likewise, the production of high titres of anti-DNP IgM antibodies indicates that the T cells have not been rendered unresponsive, as recently suggested after immunization of CD40-ligand knockout mice with allogeneic cells[5].

Although the induction of T-dependent IgM antibody responses in $CD40^{-/-}$ mice[1,2] indicates effective T cell priming, we measured the antigen-specific T-cell precursor frequencies, after immunization of the knockout mice with KLH. This revealed variations between $CD40^{-/-}$ mice with a tendency

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